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Research article

Investigation into the role of an extracellular loop in mediating proton-evoked inhibition of voltage-gated sodium channels

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ABSTRACT

Proton-evoked activation of sensory neurons is counteracted by inhibition of voltage-gated Na^+ channels, and the low acid-sensitivity of sensory neuron of the African naked mole-rat (ANMr) was reported to be due to a strong proton-evoked block of ANMrNav1.7. Here we aimed to reevaluate the role of the suggested negatively-charged motif in the ANMrNav1.7 domain IV P-loop for inhibition by protons. Patch clamp recordings were performed on the recombinant α-subunits Nav1.2–1.8. The insertion of the negatively charged motif (EKE) of ANMrNav1.7 into human Nav1.7 results in an increased proton-evoked tonic inhibition, but also in a reduced channel function. While the voltage-dependency of fast inactivation is changed in hNav1.7-EKE, pH 6.4 fails to induce a significant shift in both constructs. Proton-evoked inhibition of other channel α-subunits reveals a discrete differential inhibition among α-subunits with hNav1.7 displaying the lowest proton-sensitivity. The mutant hNav1.7-EKE displays a similar proton-sensitivity as Nav1.2, Nav1.3, Nav1.6 and Nav1.8. Overall, a correlation between proton-evoked inhibition and motif charge was not evident. Accordingly, a homology model of hNav1.7 shows that the EKE motif residues do not contribute to the pore lumen. Our data confirms that a negative charge of a postulated proton-motif encodes for a high proton-sensitivity when inserted into hNav1.7. However, a negatively charged motif is not a reliable predictor for a high proton-sensitivity in other α-subunits. Given the distance of the proton-motif from the pore mouth it seems unlikely that a blocking mechanism involving direct obstruction of the pore underlies the observed proton-evoked channel inhibition.

1. Introduction

Voltage-gated Na^+ channels (VGSC) are crucial for action potential generation in most excitable cells. In peripheral nociceptive sensory neurons, excitability seems to be finely orchestrated by several VGSC α-subunits including Nav1.1, Nav1.3, Nav1.6, Nav1.7, Nav1.8 and Nav1.9 [15]. While all of these α-subunits have been shown to carry out specialized and partially non-overlapping functions in nociceptors leading to certain types of pain, the tetrodotoxin-sensitive α-subunit Nav1.7 have emerged as a key determinant for peripheral pain signaling [3]. Human subjects carrying inherited non-functional variants of Nav1.7 never experienced the sensation of pain, whereas several gain-of-function mutations of Nav1.7 results in chronic pain syndromes [5]. Thus Nav1.7 is required for intact pain sensitivity in both rodents and humans, and an inhibition of Nav1.7 likely results in pain reduction.

Tissue acidosis develops in course of inflammation as well as ischemia, and high concentrations of protons are known to evoke pain by activating ion channels such as TRPV1, TRPA1 and acid sensing ion channels (ASICs) in sensory neurons resulting in inflammatory pain or ischemic pain [2,6,10]. Conversely, protons are also known to directly inhibit VSGCs in a concentration-dependent manner [7,13,14]. Hence, acid-evoked activation of sensory neurons and resulting pain seems to be counteracted by a concomitant reduction of Na^+ currents which are required for the generation and propagation of action potentials. Saying this however, there is a remarkable scarcity of reports which explored the effects of protons on VGSCs expressed in sensory neurons. In cultured trigeminal ganglion neurons, Nakamura and colleagues reported that acid inhibits tetrodotoxin-resistant Na^+ currents more strongly than tetrodotoxin-sensitive currents [13,14]. Whether or not this difference is due to differential acid-evoked block of specific α-subunits however, was not yet studied. Thus, the properties and relevance of proton-evoked block of the tetrodotoxin-sensitive α-subunit Nav1.7 in mammalian sensory neurons remain to be elucidated. However, tt has been suggested that the failure of protons to robustly activate sensory neurons from the African naked mole-rat (ANMr) is due to a potent proton-evoked block of ANMrNav1.7 [18]. As the ANMr lives

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underground under conditions of high CO_2 that produces tissue acidosis, it was suggested that the Nav1.7-dependent proton-evoked block of sensory neurons enables ANMr to live at such conditions without suffering from acid-evoked pain. When comparing ANMrNav1.7 with the less proton-sensitive human Nav1.7 orthologue, Smith and colleagues proposed that the amino acids EKE (position 1718–1720) in the domain IV S5–S6 extracellular linker of ANMrNav1.7 are likely to form a negatively-charged motif for protons [18]. In hNav1.7 the corresponding motif carries a 2-fold positive charge (KKV, *i.e.* + + 0), and the insertion of the EKE-motif into hNav1.7 indeed resulted in a strongly potentiated proton-evoked block. While the original report of Smith and colleagues was highly recognized at the time of publication, the finding has not been further pursued or even confirmed in later studies. In the present *in vitro* study on recombinant α-subunits, we therefore asked if the mutant construct hNav1.7-EKE containing the suggested motif for protons in ANMrNav1.7 indeed displays an increased proton-evoked block as compared to wildtype hNav1.7 We also explored to what extent protons differentially inhibit mammalian orthologues of the α-subunits Nav1.2, Nav1.2, Nav1.3, Nav1.4, Nav1.5, Nav1.6, Nav1.7 and Nav1.8, and if this differential block correlate with the charge of the proposed motif for protons.

2. Material and methods

2.1. Transfection and cell culture

Human-embryonic-kidney cells stably expressing rat Nav1.2, rat Nav1.3, rat Nav1.4 and human Nav1.5 were grown under standard cell-culture conditions with 5% CO_2 at 37 °C in Dulbecco's modified Eagle medium (DMEM, GIBCO-Invitrogen, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biochrom, Germany), 1% penicillin/streptomycin (GIBCO-Invitrogen, Germany). We added 1% G418 for stable expression of $Na_v1.2$ and $Na_v1.3$ and 0.4% zeocin (GIBCO-Invitrogen, Germany) for Nav1.5 to the medium. For $Na_v1.4$ the medium was supplemented with 2% G418. Transient transfections for human Nav1.6 and Nav1.7 as well as rat Nav1.8 were performed using the nanofectin transfection kit as described previously [19]. The mutant construct hNav1.7-EKE was generated by site directed mutagenesis of hNav1.7 cDNA using the quikchange lightning site-directed mutagenesis kit (Agilent, Waldbronn, Germany) according to the instructions of the manufacturer. The mutant was subsequently sequenced to verify intended amino acid exchanges, and to exclude further channel mutation. Nav1.6 and Nav1.8 express poorly in HEK-cells and were therefore examined in the neuroblastoma-cell line N1E115. As these cells express endogenous sodium channels, the experiments on Nav1.6 and Nav1.8 were performed in presence of 300 nM tetrodotoxin. While wildtype Nav1.6 is tetrodotoxin-sensitive, we used a mutant Nav1.6 construct being tetrodotoxin-resistant (a generous gift from Angelika Lampert, Aachen). Neuroblastoma N1E115 cells were cultured in DMEM supplemented with 10% FBS (Biochrom, Germany), penicillin/streptomycin (1%, GIBCO-Invitrogen). Plasmid encoding green-fluorescent-protein was co-transfected for the purpose of visualizing transfected cells. Transfected cells were used within 2 days.

2.2. Solutions

We performed whole-cell patch clamp recordings with an extracellular solution consisting (mmol/l): 140 NaCl, 3 KCl, 1 CaCl2, 1 MgCl2, 1 CaCl2 and 10 HEPES (or 10 MES fo acidic solutions). The pH-value was adjusted the desired pH-values with HCl or TMA-OH. For recordings of Nav1.6 and Nav1.8, 300 nM tetrodotoxin (TTX, Alomone labs, Jerusalem, Israel) was included in all solutions in order to block all endogenous TTX-sensitive Na^+ channels expressed in N1E115 cells. For the experiments we replaced the medium of the cell culture dishes with the extracellular solution (pH 7.4) and applied the test-solutions in increasing pH-values using a self-made, gravity driven application system. The pipette solution contained (mmol/l): 140 CsF, 10 NaCl, 1 EGTA, 10 HEPES. The pH-value was adjusted to 7.4 with CsOH. All solutions were stored at 7 °C in light-excluding bottles and used within one month.

2.3. Patch clamp technique and data acquisition

All experiments were performed at room temperature utilizing an EPC10 amplifier (HEKA Instruments Inc., NY, U.S.A.). We prepared and heat polished patch pipettes from glass capillaries (Science Products, Hofheim, Germany) on a micropipette puller system P-1000 (Sutter Instrument, Novato, U.S.A.) to give a resistance of 1.8-2.3 MΩ when filled with pipette solution. The series resistance was minimized by compensation of the series resistance in a range between 60 and 80% and capacitance artefacts were eliminated using the amplifier circuitry. Except for use-dependent block, linear leak subtraction based on resistance estimates from four hyperpolarizing pulses applied before the test pulse, was used for all voltage-clamp recordings, and currents were filtered at 5 kHz. We used the Patchmaster v20 × 60 software (HEKA Instruments Inc., NY, U.S.A.) for recording of the experiments, the Pulse and Pulse Fit software (HEKA Instruments Inc., NY, USA) for analysis and Origin 7.0 (Microcal Software,v Northampton, MA) for statistical analysis and curve fitting. The half-maximal effective concentration (IC_{50}) was evaluated by normalizing the peak currents amplitudes at each drug concentration value achieved in control solution. Data were fitted with Hill equation (y = ymax * (IV50n/IC50n * Cn), where ymax is the maximal amplitude, IC50 the concentration at which y/ymax = 0.5, and n is the Hill coefficient). To investigate the inactivation curves, peak currents evoked by a test pulse were measured, normalized and plotted against the conditioning pre-pulse potential.

2.4. Statistical analyses

Statistics were calculated using Origin 8.5 (Microcal Software,v Northampton, MA). Multiple comparisons for groups with equal, balanced sample sizes were performed by ANOVA with a Tukey post hoc test. If appropriate, single comparisons of independent groups of data were calculated with the unpaired *t*-test. Significance was assumed for $p < 0.05$. All data were presented as mean ± S.E.M.

2.5. Homology modeling

A homology model of hNav1.7 (UniProt accession Q15858) was generated using the 3.8 Å resolution structure of the *Periplaneta americana* sodium channel NavPaS (PDB code 5 × 0 M) [16]. Sequences were aligned using Clustal Omega [17]. 50 starting models were generated using MODELLER [4]. The internal scoring function of MODELLER was used to select 10 models, which were visually inspected and submitted to the VADAR webserver [21] to assess stereochemistry in order to select the best final model. Figures were produced using ALINE [1] and PyMOL (DeLano Scientific, San Carlos, CA, USA).

3. Results

3.1. Proton-evoked inhibition is enhanced in the hNav1.7-EKE mutant

We first examined tonic inhibition of wildtype hNav1.7 and the mutant hNav1.7-EKE by protons. Cells were held at −120 mV and 10 ms long test pulses to 0 mV were applied every 10 s. As is demonstrated in Fig. 1A and B, protons evoked a concentration-dependent block of both wildtype hNav1.7 (A) and the hNav1.7-EKE mutant (B). The resulting K_i values as calculated by the Hill equation gave pH 5.8 ± 0.05 (Hill coefficient 1.1 ± 0.06, n = 8) for wildtype, and 6.2 ± 0.1 (Hill coefficient 1.1 ± 0.04; n = 8) for hNav1.7-EKE (Fig. 1C). Although these data indicate that hNav1.7-EKE is indeed